Binding studies of E-Cadherin peptides to the EC1 domain of E-Cadherin

By

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Chairperson Dr. Teruna J Siahaan

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Abstract

The blood brain barrier (BBB) is a key role in delivering medication to the brain; if the drug molecules can overcome this obstacle, many brain diseases (i.e., Alzheimer's, Parkinson's, and brain tumor) can be effectively treated. E-cadherin is important in forming cell-cell adhesion in intercellular junctions of the biological barriers (i.e., intestinal mucosa and BBB). Cadherin peptides (HAV6 and ADTC5) have been shown to modulate the BBB in the cell culture and in in-situ rat brain perfusion models. The hypothesis is that cadherin peptides modulate the tight junctions of the BBB by binding to the EC1 domain of E-cadherin. Thus, the objective of this project is to determine the dissociation constants (Kds) of linear and cyclic cadherin peptides (i.e., HAV- and ADT-peptides) to the expressed EC1 domain of E-cadherin using circular dichroism (CD) spectroscopy. The data show that the cyclic HAVc3 peptide (Kd = 66.7±18.0 nM) has better binding property to the EC1 domain than the linear HAV6 peptide (Kd = 120.1±11.9 nM). Cyclic ADTC5 (Kd = 50.2± 11 nM) has lower dissociation constant than ADTC1 (Kd = 119.7± 16 nM). Mutation of the valine residue in ADTC5 to glutamic acid (ADTC7; Kd = 0.43± 0.050 nM) and threonine (ADTC8; Kd = 0.45±0.012) makes the peptides to have 100-fold tighter binding compared to the EC1 domain. Mutation of the valine residue in ADTC5 to a tyrosine residue (ADTC9; Kd = 0.038± 0.009) increases binding to the EC1 domain by 1000-fold. Finally, mutation of the valine residue to arginine demolishes the activity of ADTC10. In the future, the activity of ADTC9 to modulate the BBB will be compared to ADTC5 in in vitro and in vivo models of the BBB.

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1. Introduction

The blood brain barrier (BBB) consists of 100 billion capillaries with a combined length of about 650 km; the surface area of these capillaries is approximately 20 square meters [1]. These capillaries have a dynamic function to both allow and prevent molecules from entering the brain [1-3]. Unfortunately, this means that the BBB also limits many beneficial medications from accessing the brain to treat diseases such as Alzheimer's, Parkinson's, brain tumors, and multiple sclerosis. However, the drug molecules are able to pass through the BBB using the paracellular and transcellular pathways [1-5]. The transcellular pathway is via passive diffusion, passive diffusion modified by efflux pumps, or active transport mechanism [3]. The transcellular passive diffusion mechanism engages several steps, including partition of the drug into cell membranes followed by entrance into the intracellular space. The drug then penetrates cell membranes of the BBB and enters the brain tissue. For partitioning into the cell membranes, the physicochemical properties of the drug become essential. Some drugs are recognized by efflux pump; although they effectively partition to the cell membranes, the efflux pumps expel them from the cell membranes that prevent them from crossing the BBB. A fraction of drug molecules cross the BBB using the active transport mechanism, which is normally an ATP-energy-dependent process. In

contrast, small ions and hydrophilic molecules cross the BBB using the paracellular pathway or penetrate via the intercellular pathway. The molecule permeation through the paracellular pathway is limited due to the presence of tight junctions (zonula occludens) between the opposing membranes of endothelial cells of the BBB [1-4]. Tight junctions are normally found in tissues that have absorption functions and impose barricades between the blood and other tissues. The tight junctions are connected by several transmembrane proteins, including occludins, claudins, and junctional adhesion molecules (JAMs) [6-13]. The adherens junctions (zonula adherens) are found underneath the tight junctions and desmosomes, which are below the adherens junctions [3, 4]. Calcium-dependent proteins in the cadherin family are involved in cell-cell adhesion in the adherens junctions and desmosomes [14, 15]. E- and VE-cadherins mediate cell-cell adhesion between the adherens junctions of the BBB while desmocollins and desmogleins in the cadherin family form cell-cell adhesion in the desmosomes [16, 17].

Cadherins help to hold and connect the cells together; they are also involved in cell morphogenesis, embryonic cell growth and separation [18], and cell migration [19]. Different cadherins are found in different tissues; for example, N-cadherin is involved in synapse formation in the neurons [18] while P-cadherin is found in the placenta [20]. Cadherins are transmembrane glycoproteins with extracellular, membrane, and cytoplasmic

domains [19]. The extracellular fragments of cadherins can be divided into five EC domains (EC-1 to EC-5). Each EC domain has 100–110 amino acid sequences [21]. The cell adhesion behavior of cadherin is calcium-dependent, and the removal of calcium eliminates the cell-cell adhesion activity [22-24]. Calcium ions bind at the inter-repeat domain of the extracellular portion of E-cadherin; for example, three calcium ions are found at the inter-domain between the EC1 and EC2 domains. In the absence of calcium, the EC domain of cadherin undergoes a conformational change from a rod-like shape to a globular shape [21].

Cadherin-mediated cell-cell adhesion is a dynamic and reversible process that allows penetration of some molecules through the junctions. Modulation of cadherin interactions has been shown to temporarily enhance the permeation of paracellular marker molecules (i.e., ^{14}C-mannitol) and an anticancer drug (^{3}H-daunomycin) through the paracellular pathway of the in vitro and in vivo models of the BBB [24-26]. Many molecules cannot readily pass through the limited pore sizes of the tight junctions, which allow only molecules with hydrodynamic radii less than 11 Å to permeate [4]. If a way is found to increase the permeation of drug molecules through the tight junctions of the BBB, it may help in delivering many drugs to treat brain diseases. Many methods have been investigated to modulate the tight junctions for improving paracellular permeation of molecules. EDTA, EGTA, and citrate open the tight junctions non-selectively by chelating calcium and enhance

passive permeation of molecules through the paracellular pathways of MDCK and Caco-2 cell monolayers [25]. Palmitoyl carnitine, deoxy- or glycol-cholate, and chitostan have also been known to increase the transport of molecules through the BBB [25]. Another method to modulate cell-cell adhesion involves inhibiting the protein kinase activity to phosphorylate of the cytoplasmic domain of occludin, claudin, and cadherins. Desphosphorylation of the intercellular junction proteins results in loosening of cell-cell adhesion due to protein translocation of these protein from the cell surface into the cell intracellular compartments [4]. However, it is difficult to control dephosphorylation of intercellular junction proteins without affecting other proteins as well. Finally, a hypertonic solution of mannitol has been used to open the tight junctions and deliver anticancer drugs to treat brain tumors; this hypertonic solution presumably causes crenation of the microvessel endothelial cells to loosen the tight junctions [27-29].

The approach in our laboratory is to temporarily disrupt the cadherin-cadherin interactions to increase the openings in the tight junctions for paracellular permeation of molecules through the BBB [2, 25]; this disruption is caused by cadherin peptides. The peptides were designed from the contact regions of the EC1 domain of E-cadherin (Table 1) [24]. His-Ala-Val (HAV)- and Ala-Asp-Thr (ADT)-peptides (Table 2) were found to modulate the intercellular junctions of MDCK and Caco-2 cell monolayers as determined by

the change in trans-electrical epithelial resistance (TEER) values [24, 26]. Compared to control peptides and vehicle, HAV6 and ADTC5 peptides increased the paracellular permeation of ^{14}C-mannitol through the BBB in an in-situ rat brain perfusion model in a concentration-dependent manner [25, 30]. The fluorescence-labeled HAV and ADT-peptides decorated the intercellular junction proteins of bovine brain microvessel endothelial cells (BBMEC) [16] and MDCK [24] monolayers, indicating that the peptides bind to the intercellular junctions proteins. The hypothesis is that HAV- and ADT-peptides modulate the intercellular junction by binding to EC domain(s) of cadherins and inhibit cadherin-cadherin interactions. One way to test this hypothesis is to study the binding properties of the peptides to the expressed extracellular domains of E- and VE-cadherins. Previously, NMR and CD spectroscopy studies showed that HAV6 peptide binds the EC5 domain of E-cadherin [31]. However, there is no evidence that the cadherin peptides bind to other EC domains (EC1 to EC4) of E-cadherin; the hypothesis is that a cadherin peptide binds to different EC domains with different binding constants. Furthermore, there is no direct evidence that HAV- and ADT-peptides bind to VE-cadherin and, most likely, a cadherin peptide has different binding constants to the EC1 domain of E- and VE-cadherins. In the future, the results from this study will be used to design peptides selective to E-cadherin or VE-cadherin.

The long-term goal of this study is to compare the binding properties of HAV- and ADT-peptides to the EC1 domains of E- and VE-cadherins. The short-term goals of this project are (a) to express and purify the EC1 domain of E- and VE-cadherins and (b) to determine the dissociation constants (K_ds) of HAV- and ADT-peptides to the EC1 domain of E-cadherin. In this work, we have successfully expressed and purified the EC1 domain E-cadherin. Although the EC1 domain of VE-cadherin can be expressed, the purification of this protein is still difficult. Thus, only binding studies between the EC1 domain of E-cadherin and cadherin peptides were carried out; these were done using circular dichroism (CD) spectroscopy. The results indicated that different peptides bind to EC1 domain of E-cadherin with different dissociation constants (K_d) and these data will be used in an effort to improve the affinity of E-cadherin peptides to E-cadherin over VE-cadherin.

2. Experimental Procedures

2.1. Cloning EC1 of E- and VE-cadherins cDNA into plasmid pASK-IBA6

cDNA sequences of E- and VE-cadherins were taken from human epithelial and endothelial cadherin genomes, respectively (Table 1). The genes for EC1 domains of E- and VE-cadherins were inserted into pASK-IBA6 plasmid (IBA, Gottingen, Germany) [32]. The pASK-IBA6 plasmid has 3681 DNA base pairs (bp); it carries AmpR, f1 origin, and Multi

Cloning Sites 1 (MCS 1) gene regions. The MCS 1 region consists of Streptag-I, Factor Xa, and EC1 genes. The N-terminus of Streptag-I (WSHPQFEK) was connected to the C-terminus of the OmpA signal sequence on the pASK-IBA6; the OmpA signal is used to direct the fusion of EC1 into the periplasm of the host cells.

Table 1. The protein sequences of the EC1 domains of E- and VE-cadherin

EC1 Domain of E-Cadherin	EC1 Domain of VE-Cadherin
WSHPQFEKIEGRDWVIPPISCPE NEKGPFPKNLVQIKSNKDKEGK VFYSITGQGADTPPVGVFIIERE TGWLKVTEPLDRERIATYTFSH AVSSNGNAVEDPMEILITVTDQ NDNKPEFTQEVFKGSVMEGAL PGTSVMEVTATDADDD	DWIWNQMHIDEEKNTSLPHHV GKIKSSVSRKNAKYLLKGEYVG KVFRVDAETGDVFAIERLDRENI SEYHLTAVIVDKDTGENLETPSS FTIKVHDVNDNWPVFTHRLFNA SVPESSAVGTSVISVTAVDADDP

The strep-tag sequence was added to provide a single step protein purification using StrepTactin II affinity column chromatography. It has been shown previously that addition of the strep-tag sequence does not interfere with the folded structure and function of the EC1 domain of E-cadherin. The cDNA of pASK-IBA6/EC1 was transformed into DH5α competent cells, and the cells were grown on LB agar plates with 100 mg/mL ampicillin at 37^{o}C overnight. Then, the cells were subcultured in 5 mL LB medium containing 100 mg/mL ampicillin and

incubated at 37^{o}C overnight to amplify the plasmid DNA; the Qiagen Spin Miniprep Kit (Stratagene) was used to isolate the plasmid DNA.

This plasmid DNA (pASK-IBA6/EC1 DNA) was transformed into BL21 cells using the heat shock method. The plasmid (1 µL) was transferred to 50 µL of BL21 cell suspension, and mixed by using vortex. To create the heat shock to allow the plasmid DNA to enter the cells, the mixture was incubated in the following sequential conditions: (a) ice for 30 min, (b) 42^{o}C water bath for 30 sec, and (c) ice for 3 min. 200 µL of SOC medium **(**1.55 g yeast, 0.25 mL of 1 M KCl, 0.5 mL of 1 M $MgCl_2$, 0.5 mL of 1 M $MgSO_4$, 1 mL of 1 M glucose in distilled H_2O) was added into the cells under sterile conditions. The mixture was placed in a shaking incubator for 1 h. After 1 h, 50 µL and 150 µL of the BL21 cell aliquot were spread separately on two different LB agar plates containing 100 mg/mL ampicillin; this was followed by overnight incubation at 37^{o}C. The survival colonies were observed on the plates as cells containing the EC1 gene that is resistant to ampicillin. A single colony of BL21 cell from LB agar was selected and transferred into 4 × 5 mL of LB medium containing 1000× of 10 mg/mL ampicillin. The cells were incubated overnight in the orbital shaker at 250 rpm and 37^{o}C. These cells were prepared as mini-cultured growth to detect production of the EC1 protein.

2.2. Overexpression of the EC1 protein

The BL21 cells from the mini culture (see above) were added into 4 × 250 mL of the LB medium (NaCl 10 g, peptone 10 g, yeast extract 5 g) containing 1000× of 100 mg/mL ampicillin. They were incubated at 37^{o}C in the shaker incubator until the cell mixture reached an OD_{550} (optical density, at wavelength 550) of 0.5–0.6. After this, 25 µL of anhydrotetracycline was added to induce protein production followed by incubation for another 6 h at 30^{o}C. Then, the cells were harvested and centrifuged at 4,500 rpm for 10 min at 4^{o}C. The pellets were isolated and stored at –80 ^{o}C until they were used in the next process.

2.3. Purification of recombinant EC1

For protein isolation, the cell pellets were resuspended at pH 8 in a binding buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.02 % (w/v) NaN_3). The cells were lysed with a French Press machine three times to release the EC1 protein. Cell debris was removed by centrifugation at 21000 × g for 1 h at 4^{o}C, and the supernatant containing the protein was collected. The binding buffer was added to the supernatant and it was centrifuged again at

4500 rpm for 20 min to equilibrate the protein. 4 mL of the EC1 domain solution was loaded into a StrepTactin II chromatography column for affinity purification. The column was washed with 10 × column volume (50 ml) of binding buffer to elute all the proteins except the EC1 protein. The pure EC1 protein was then eluted from the column with 6 × column volume (30 ml) of elution buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.02 % (w/v) NaN_3, 2.5 mM DTT, 5 mM desthiobiotin) at pH 8 and collected in several fractions [33]. The pure protein fractions were pooled and concentrated using concentrator membrane with 10 a kDa MW cutoff (EMD Millipore, Billerica, MA). SDS PAGE was run after purification to visualize the pure EC1 protein.

Table 2. The amino acid sequences of the modified cyclic peptides and the K_d of binding of each peptide to the EC1 domain of E-cadherin			
Peptide Name	**Sequence**	**Molecular Weight**	**Kd (nM)**
Linear HAV6	Ac-SHAVSS-NH_2	627.66	120.1±11.9
Cyclic HAVc3	Ac-CSHAVC-NH_2	657	66.7±18.0
ADTC1	Ac-CADTPPVC-NH_2	844	119.7± 16
ADTC5	Ac-CDTPPVC-NH_2	744	50.2± 11
ADTC7	AC-CDTPPEC-NH_2	802.89	0.43± 0.050
ADTC8	Ac-CDTPPTC-NH_2	774.88	0.45±0.012
ADTC9	Ac-CDTPPYC-NH_2	836.95	0.038± 0.009
ADTC10	Ac-CDTPPRC-NH_2	829.96	Not active

2.4. Peptides

The peptides used to titrate the EC1 domain are shown in Table 2. They were synthesized with C-terminal carboxyamide and N-terminal acetyl group using the solid phase method with Fmoc-protected amino acids in an automated peptide synthesizer. The peptides were cleaved from the resin with trifluoroacetic acid (TFA) in the presence of scavengers (i.e., anisol and ethane dithiols (EDT)). The disulfide bond in cyclic peptide was formed by air oxidation of the parent linear peptide in sodium bicarbonate buffer at pH 9.0. The peptides were then purified using a C18 semi-preparative reversed-phase HPLC column

and the pure fractions were analyzed by analytical HPLC with a C18 column. The pure peptides were pooled and lyophilized; the purity of all peptides was higher than 95%. Mass spectrometry was used to determine the identity of each peptide.

2.5. Circular Dichroism (CD)

CD spectrometry was used to evaluate the binding properties of cadherin peptides to the EC1 domain of E-cadherin [34]. In this case, the EC1 protein was titrated with different concentrations of peptide followed by observing the spectrum of the protein after each peptide addition. The spectral changes were plotted against peptide concentrations to determine the peptide dissociation constant (K_d). Because the phosphate buffer does not interfere with the protein and peptide spectra, the EC1 protein was dialyzed into phosphate (KH_2PO_4) buffer for 1.5 h before CD experiments. Prior to conducting the experiment, the CD instrument was equilibrated for 15 min. First, the CD spectra of the EC1 protein at different concentrations (i.e., 0.01, 0.005, 0.0025, 0.00125, 0.000625 mM) were scanned. The optimal concentration for the binding studies was found to be 0.01 mM, which gives a distinct spectrum of the EC1 domain, as observed previously [35, 36]. Then, the EC1 protein (500 μL) was titrated with every 5 μL of buffer up to a total addition of buffer of 100 μL; this was done to see whether the addition of buffer and the dilution process dramatically

affect the EC1 spectra. In every experiment, the contributions of peptide and buffer to the spectrum of the EC1 were subtracted to acquire the spectrum of protein during binding.

As an example, a stock solution of the HAV6 peptide (1.59 μM, 0.001 mg/ml) was prepared in phosphate buffer at pH 7.0. After the protein was dialyzed into phosphate buffer, 500 μL of EC1 was placed in a mini CD cuvette. Then, 5 μL of stock peptide solution was added into the protein gradually; after each addition, the solution mixture was equilibrated for 15 min prior to collecting the CD spectrum. The peptide solution was added until binding saturation was observed as reflected by the lack of change in CD absorption upon further peptide addition. A similar procedure was carried out for the titration of EC1 with cHAV3, ADTC5, and ADTC1 peptides. Due to high potency of the mutated cyclic peptides (ADTC7–ADTC10), a lower concentration stock solution of peptides (0.0001 mg/mL; ADTC7 0.125 μM, ADTC8 0.129 μM, ADTC9 0.119 μM, ADTC10 0.120 μM) was used. The peptide stock solution was added to the protein every 1–2 μL per addition. The collected CD data were converted to a readable Excel data file for processing to subtract the effect of buffer and peptide from the collected spectrum. A plot of CD absorption change at 220 nm vs. peptide concentration was generated and the K_d of each peptide was calculated using a SigmaPlot program.

3. Results and Discussion

3.1. Expression and purification of the EC1 domains of VE- and E-cadherins

The EC1 domain of VE-cadherin was successfully expressed using BL21 *E. coli* cells. Unfortunately, after elution of the EC1 domain of VE-cadherin from the affinity column (StrepTactin II), there were three bands of eluted fractions (Figure 1). The highest molecular weight band (Band A, Lanes 2 and 3, Figure 1) was from the phosphorylated EC1 domain, as previously found by other investigators. Later, we found that addition of a kinase inhibitor during the expression of VE-cadherin suppressed the intensity of the phosphorylated EC1 domain (data not shown).

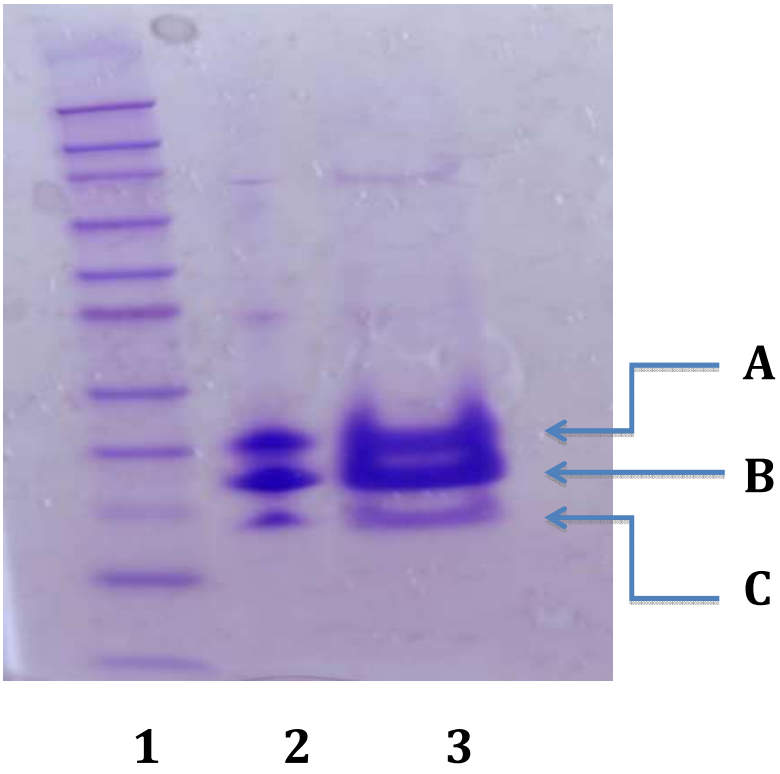


Figure 1. The SDS-PAGE of the EC1 domain of VE-cadherin after it was eluted from the affinity column chromatography using a StrepTactin-II column. **Lane 1:** The molecular weight markers. **Lanes 2 and 3**: The eluted EC1 domain of VE-cadherin at different eluted fractions. **Band A** is the phosphorylated EC1. **Band B** is the desired EC1 domain. **Band C** is the enzymatic degradation product of the EC1 domain of VE cadherin.

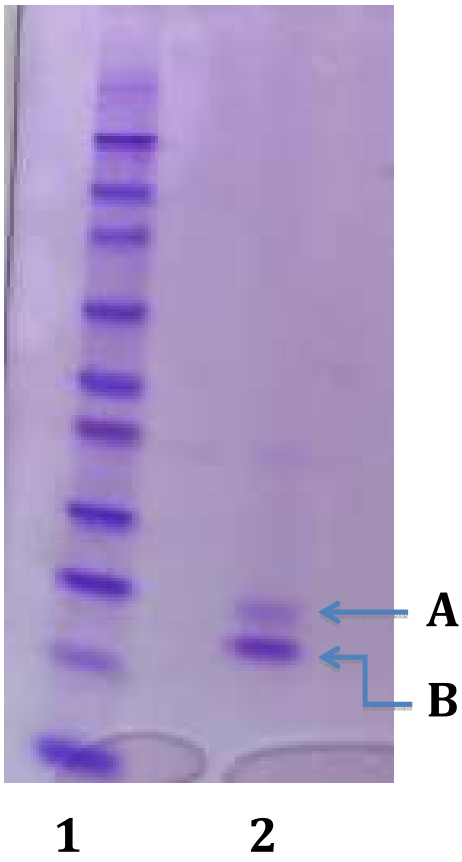


Figure 2. The SDS-PAGE of the EC1 domain of VE-cadherin after addition of a cocktail of proteolytic enzyme inhibitors during protein isolation. **Lane 1:** Molecular weight markers. **Lane 2:** The eluted EC1 domain from the StrapTactin II column. **Band A** is the phosphorylated EC1 domain. **Band B** is the desired EC1 domain of VE-cadherin.

The middle band (Band B, Figure 1) is the desired EC1 domain while the bottom band (low molecular weight band, Band C, Figure 1) is from the degradation product of the EC1 domain of VE-cadherin. Addition of a cocktail of proteolytic enzyme inhibitors during the isolation and purification of the EC1 domain eliminated the lowest band (Band C) as shown in Figure 2. However, the phosphorylated EC1 is still present in a small amount.

Due to the difficulty in purifying the EC1 domain of VE-cadherin, the focus was directed into the expression, isolation, and purification of the EC1 domain of E-cadherin. The pure EC1 from E-cadherin was used to study the binding between E-cadherin peptides and the EC1 domain. The growth of BL21 *E. coli* cells in LB medium was followed for 11 h, and the growth of the cells plateaued after 9 h (Figure 3). The overexpression of the EC1 protein was stimulated with anhydrotetracycline at after 2 h of cell incubation in LB medium. Every hour the cells were lysed and the protein content was evaluated with SDS-PAGE (Figure 4). Before induction, the cells did not produce any EC1 domain (Figure 4, Lanes 2 and 3). After induction, there was a time-dependent increase in the production of EC1 between 1 and 5 h after induction; the highest intensity was found at 5 h after induction (Figure 4, Lanes 4–8). However, beyond 5 h after induction, the amount of protein was the same. Therefore, for overproduction of the protein, the cells were harvested 5 h after induction with anhydrotetracycline. After the cells were lysed, the protein was purified using a StrepTactin

II column. The pure EC1 domain was eluted and concentrated; the purity of the EC1 domain protein is shown in Figure 5 as a single band in SDS-PAGE. Unlike the EC1 domain of VE-cadherin, the EC1 domain of E-cadherin had no enzymatic degradation product.

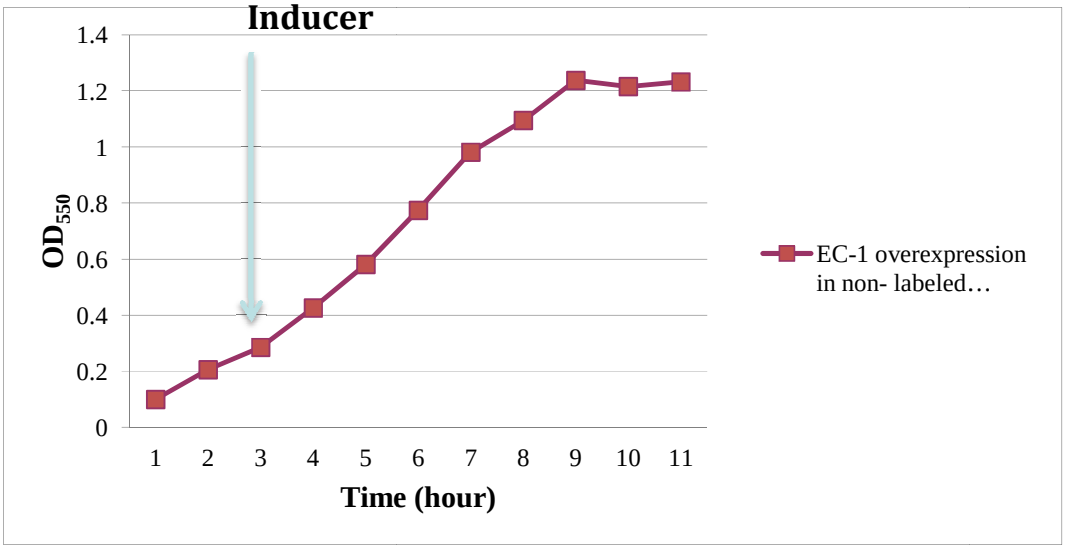


Figure 3. The diagram of cell growth of BL21 *E. coli* as measured by optical density at 550 nm *vs.* time in hours during the production of the EC1 domain of E-cadherin. The maximum growth plateaued at the 9-h time point. The anhydrotetracycline as the inducer of protein expression was added at the 2-h time point.

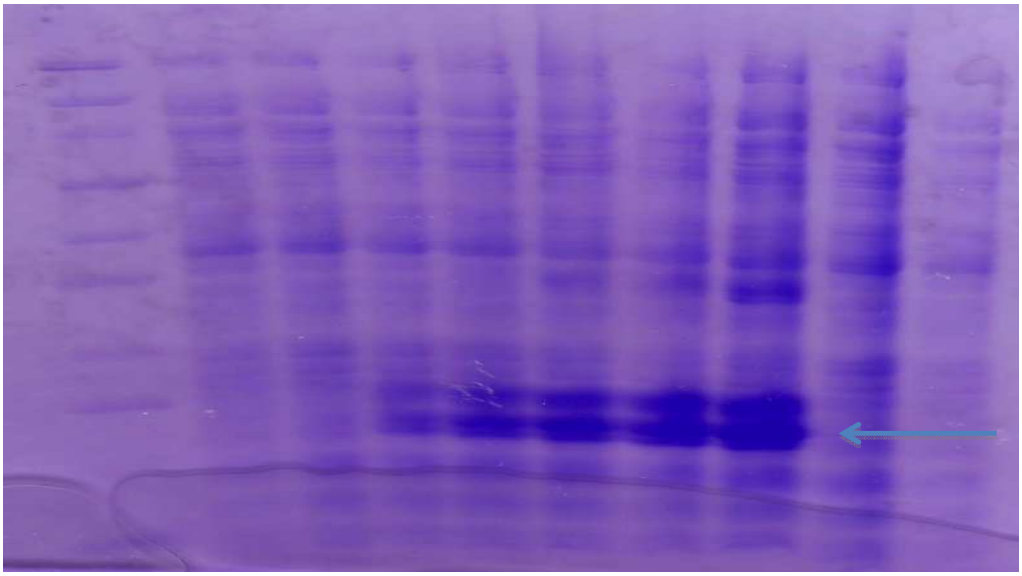


Figure 4. The SDS-P

The EC1 protein production 1, 2, 3, 4, 5, 6, and 7 h after addition of anhydrotetracycline, respectively. There is no production of protein before adding the inducer **(Lanes 2–3)**; an abundance of protein was produced after induction of protein production at the 2-h time point (**Lanes 4–8**). After passing through the StrepTactin column, all the EC1 was selectively retained in the columns and the washing buffer showed no EC1 band (**Lanes 9–10**).

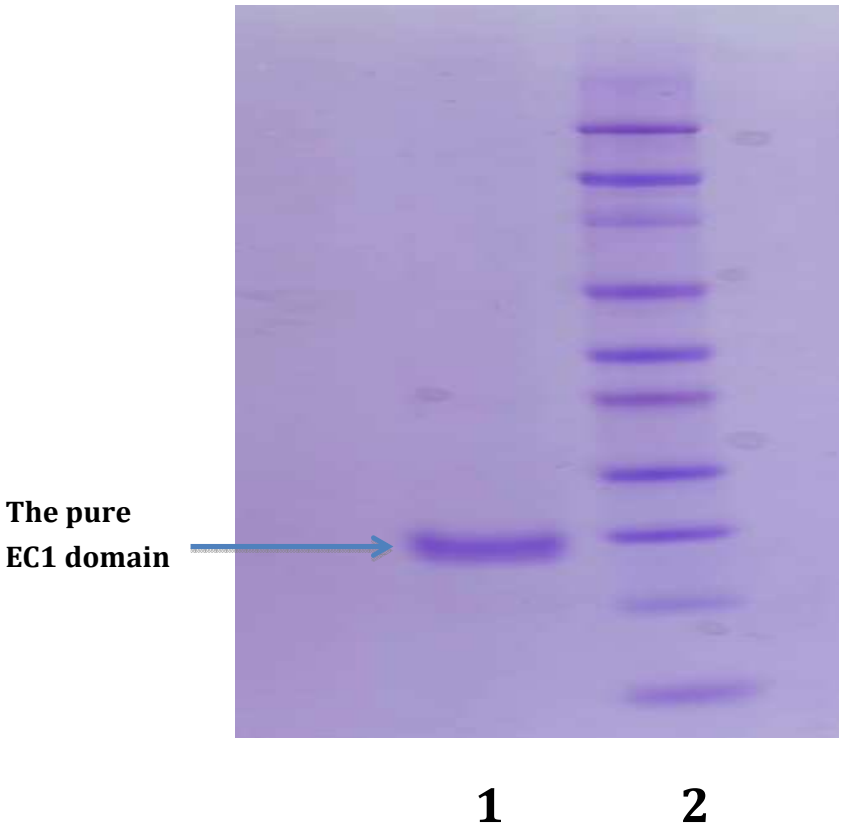


Figure 5. SDS-PAGE of pure EC1 domain of E-cadherin after elution from the affinity column (StrepTactin II). **Lane 1:** The pure EC1 domain of E-cadherin. **Lane 2:** The molecular weight marker. Unlike the EC1 domain of VE-cadherin, the EC1 domain of E-cadherin does not show any phosphorylated protein or any enzyme degradation product.

3.2. Binding studies of E-cadherin peptides with the EC1 Domain of E-cadherins

The spectra of the EC1 domain at different concentrations show a minimum at 216 nm, indicating the presence of a high amount of beta-sheet structure, which is consistent with the structure of the EC1 domain (Figure 6). The optimal concentration of the EC1 domain was found at 0.01 mM for the binding studies. The peptide titration experiments were done by gradually adding 5 μL (0.1–0.16 μM) of peptide solution up to a total of 50 μL into a 500 μL solution of the EC1 domain. Peptide binding to the EC1 domain was monitored by the change in CD absorption at 220 nm upon peptide addition. The contribution of peptide and buffer to the CD spectrum was subtracted to obtain the final protein spectrum upon complexation. The titration of the EC1 domain with buffer solution every 5 μL up to 50 μL did not dramatically change the absorption of the protein at 220 nm (Figure 7). In contrast, addition of the cyclic HAVc3 peptide dramatically changed the absorption at 220 nm, and the change plateaued at high concentrations (Figure 8). The results suggest that the peptide binds to the protein and the binding process can be saturated.

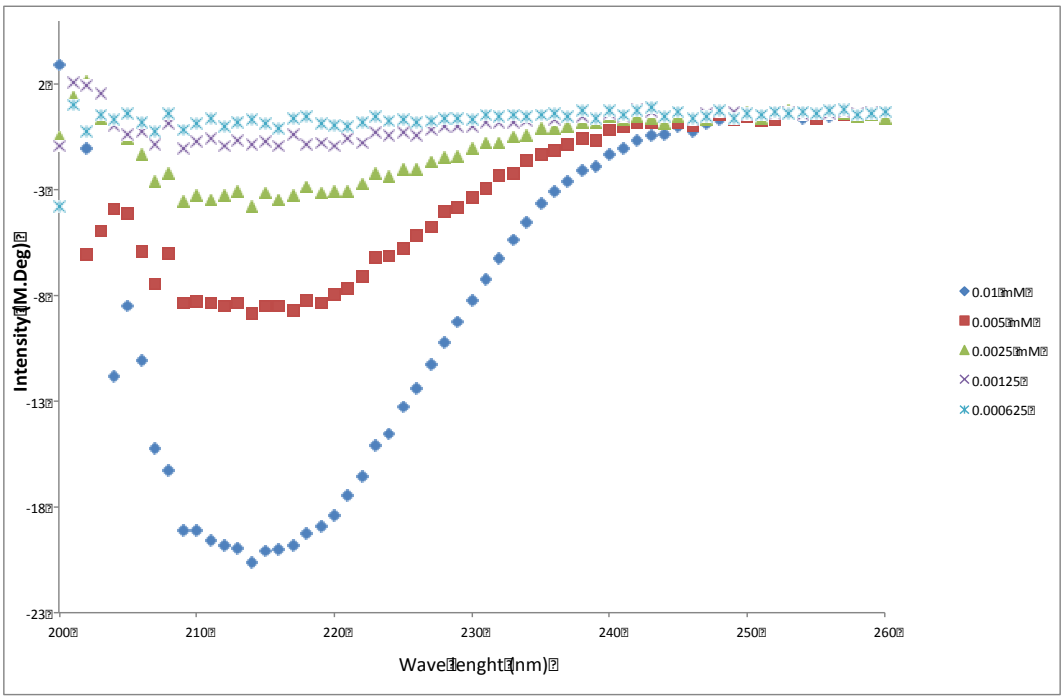


Figure 6. CD spectra of the EC1 domain of E-cadherin at different concentrations (0.000625, 0.00125, 0.0025, 0.005, and 0.01 mM) in phosphate buffer at pH 7.0. There is a concentration-dependent change in CD spectra of the EC1 domain, and the best protein concentration for the binding studies is 0.01 mM. The spectra of the EC1 domain resemble the previous CD spectrum of the EC1 domain of E-cadherin investigated in our laboratory.

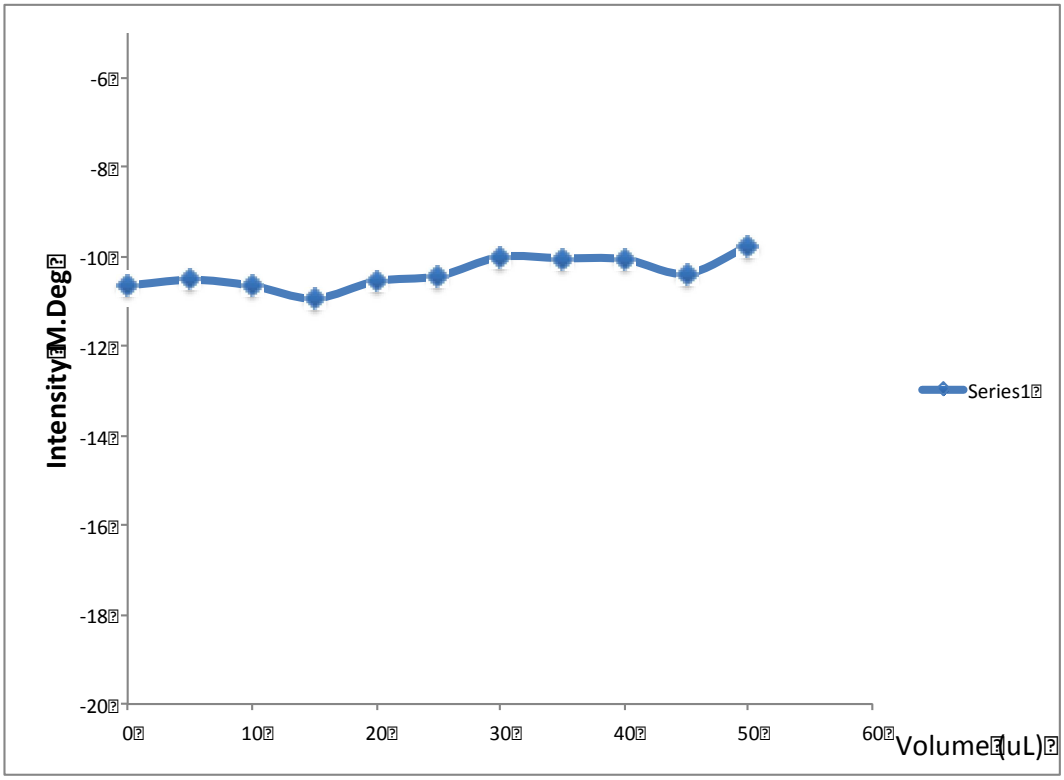


Figure 7. The effect of buffer dilution on the CD absorption of EC1 observed at 220 nm. The concentration of the EC1 domain is 0.01 mM in 500 µL phosphate (KH_2PO4) buffer; the solution was titrated with the same buffer every 5 µL up to the addition of 50 µL of buffer. There were very small changes in the intensity at 220 nm upon dilution with buffer.

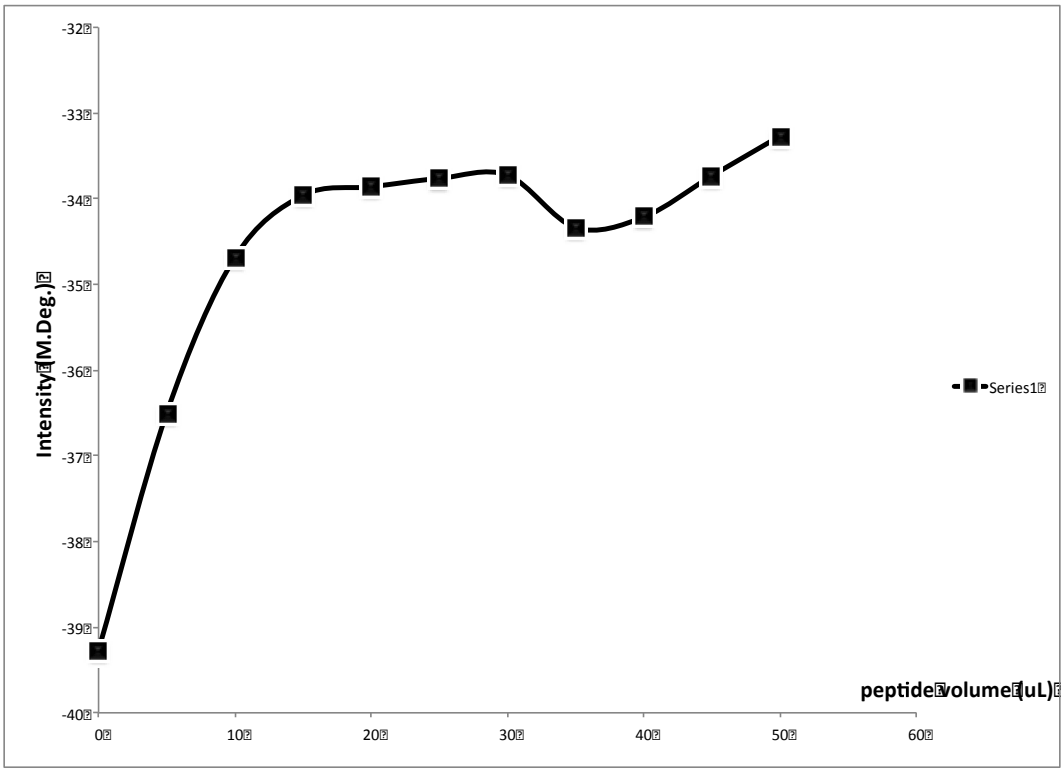
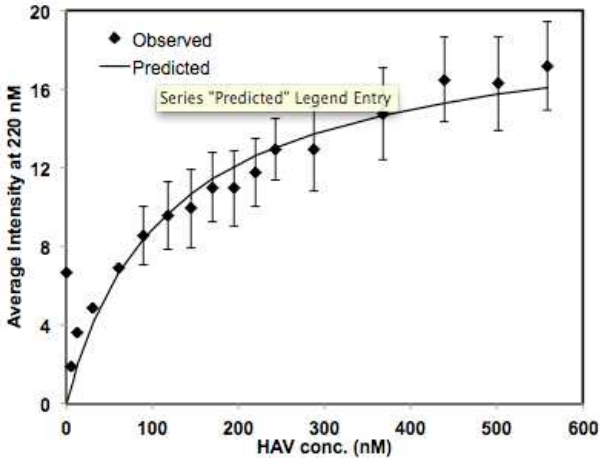


Figure 8. An example of the CD absorption changes (Y-axis; millidegree) observed at 220 nm upon titration of 0.01 mM EC1 domain in 500 μL buffer with 0.001 mM HAVc3 peptide every 5 μL up to 50 μL. The contribution of the peptide to the spectrum was subtracted from the protein spectrum. There is a dramatic change of absorption intensity of the EC1 domain at 220 nm upon addition of 5 to 15 μL of cHAV peptide. There is a plateau in the absorption change beyond the addition of 15 μL of peptide, indicating the peptide binding saturation.

A



B

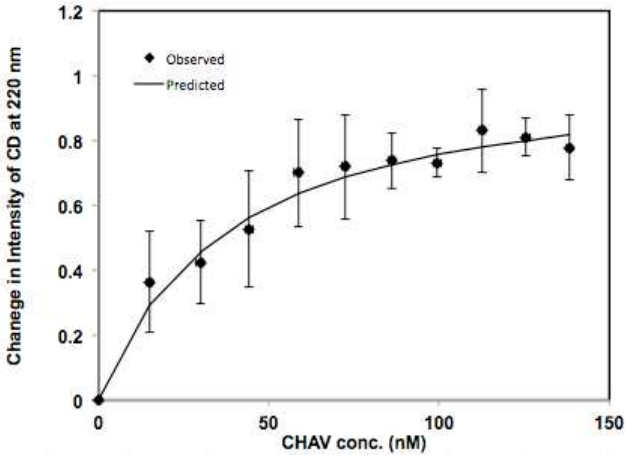


Figure 9. Plots of the change in CD intensity of EC1 (0.01 mM) at 220 nm upon addition of different concentrations of linear HAV6 peptide (**Panel A**) and cyclic HAVc3 peptide (**Panel B**). The K_d of the binding between linear and cyclic peptides to the EC1 domain was determined by SigmaPlot.

The 2D NMR studies showed that HAV6 and ADTC5 peptides bind to the expressed ^{15}N-labeled EC1 for the E-cadherin. Titration of the ^{15}N-labeled EC1 with HAV6 and ADTC5 peptides caused a shift of ^{1}H and ^{15}N resonances of several residues in the EC1 domain of E-cadherin. Thus, the binding properties of these peptides were also evaluated using CD spectroscopy. For the linear HAV6 peptide (1.59 μM), a total volume of 100 μL of peptide was added to a 500 μL solution of EC1 to reach binding saturation. The change in absorption at 220 nm was observed upon peptide addition; the change was found to be due to the conformational change of EC1 upon binding the HAV6 peptide (Figure 9A). The estimated K_d of binding of HAV6 peptide to the EC1 domain is 120 ± 11.9 nM (Table 2).

The cyclic HAVc3 peptide has a K_d of 66.7 ± 18 nM, indicating that the cyclic peptide has higher affinity for the EC1 of E-cadherin than does the linear HAV6 peptide (Figure 9B, Table 2). This result is consistent with results from the inhibition of resealing of the intercellular junctions of the MDCK cell monolayers (data not shown), and cyclic HAVc3 peptide is a better inhibitor of intercellular junction resealing than the linear HAV6 peptide. This could be due to conformational restriction of the cyclic peptide compared to that of the linear peptide.

Cyclic ADTC5 has greater activity than linear HAV6 peptide in inhibiting the resealing of the intercellular junctions of MDCK cell monolayers. In this study, cyclic ADTC5 peptide

has a K_d of 50.2±11 nM, which is better than that of the linear HAV6 peptide (K_d = 120±11.9 mM). The larger cyclic ADTC1 peptide has a higher K_d (119.7±16 nM) than does cyclic ADTC5 peptide (K_d = 50.2±11 nM), which supports our previous in vitro study. The valine residue in ADTC5 (Ac-CDTPPVC-NH_2) has been shown to be important for biological activity in modulating the intercellular junctions of MDCK monolayers. Therefore, the valine residue in ADTC5 was replaced with the Glu (E), Thr (T), Tyr (Y), and Arg (R) residues to give ADTC7, ADTC8, ADTC9, and ADTC10, respectively. Both ADTC7 (K_d = 0.43± 0.050 nM) and ADTC8 (K_d = 0.45±0.012 nM) have 100-fold tighter binding affinity to the EC1 domain of E-cadherin than does the parent ADTC5 (50.2±11 nM). The binding study shows that ADTC9 has the best affinity (K_d = 0.038±0.009 nM) to the EC1 domain of E-cadherin; it has ten-fold higher affinity than ADTC7 and ADTC8. The ADTC10 peptide has no binding affinity for the EC1 domain, which suggest that mutation of the valine residue to the lysine residue eliminates the peptide binding affinity to the EC1 domain. This result reaffirms the importance of the residue at the valine position of cyclic ADT peptides.

4. Conclusion

The binding studies using CD spectroscopy confirm the NMR studies showing that HAV6 and ADTC5 peptides bind to the EC1 domain of E-cadherin. This study also shows that cyclic HAVc3 peptide has a higher binding affinity than does the linear HAV6 peptide, which is consistent with the in vitro activity study using MDCK cell monolayers. Binding studies of cyclic ADT peptides found that cyclic ADTC9 peptide (K_d = 0.038±0.009 nM) has 1000-fold lower K_d than ADTC5 peptide (50.2±11 nM), indicating that mutation of the Val residue to the Tyr residue enhances peptide binding. In the future, the concentration-dependent activity of ADTC9 peptide will be compared to that of ADTC5 using in vitro and in vivo BBB models.

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